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**14. ABSTRACT**

In mammals and other higher eukaryotes, SRA-RING proteins are essential for global maintenance of DNA methylation. This research project focuses on the discovery of new ubiquitination targets for the Arabidopsis SRA-RING protein VIM1 and the human SRA-RING protein UHRF1. Additionally, it will closely examine the methylcytosine-binding specificity of UHRF1, with a specific focus on non-CpG contexts. The proposed work is ongoing, and so far the major accomplishments include creation of relevant plant lines and development of in vitro assays.

**15. SUBJECT TERMS**
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## INTRODUCTION

DNA methylation is an important regulator of genome function, and disruptions in DNA methylation play a role in many types of cancers. Several studies have identified specific DNA methylation patterns as prognostic markers for breast cancer[1-4]. In mammals and other higher eukaryotes, SRA-RING proteins are essential for global maintenance of DNA methylation[5,6]. These proteins also regulate cellular processes relevant to breast cancer pathology, including gene expression and the cell cycle[7-10]. Each protein in the SRA-RING family contains a methylcytosine-binding SRA (SET- and RING-associated) domain, a PHD domain, and one or more RING domains. Studies in mammalian cell lines have shown that the RING domain of ICBP90/UHRF1, a SRA-RING protein that is often misregulated in cancers, can target core histones or DNMT1 for ubiquitination[11,12]. Although these studies have provided valuable insight into the function of UHRF1 in epigenetic regulation and heterochromatin structure, they have not exhaustively considered other potential substrates for UHRF1 ubiquitin ligase activity. A significant portion of my research project focuses on the discovery of new ubiquitination targets for the Arabidopsis SRA-RING protein VIM1, a UHRF1 ortholog with a similar domain structure and comparable roles in epigenetic regulation (Fig 1). I will follow up on these studies with in vitro experiments involving UHRF1 aimed at further understanding the substrates and specificities of its RING and SRA domains. This research will provide details on fundamental epigenetic mechanisms that are central to the molecular pathology of breast cancer.


**Figure 1**. Arabidopsis VIM1 and human UHRF1 proteins share a similar domain structure, with each containing a PHD domain, an SRA domain, and at least one RING domain.

## BODY

Here, I discuss research accomplishments associated with a revised Statement of Work (SOW) recently submitted to the Cornell University Office of Sponsored programs. DOD approval of this SOW is pending.

### Task 1: Investigate the role of the VIM1 RING domain in epigenetic regulation in *Arabidopsis thaliana*

1. Identify substrates for VIM1 E3 ubiquitin ligase activity using a comparative proteomics approach in wild-type plants and RING domain mutants (Months 1 - 18)

This work is in early stages, with the main accomplishment so far being the creation of relevant plant lines. These were made in a *vim1 -/-* T-DNA insertion mutant background. I have transformed these mutants with T-DNA constructs for expression of the *VIM1* gene under its native promoter. Through site-directed mutagenesis, point mutations have been made in zinc-chelating residues of one or both of the VIM1 RING domains in these expression constructs (Fig. 2). Thus, the only form of VIM1 expressed in these plant lines will be a version containing point mutations that should abolish RING domain activity.

I intend to use these plant lines in future studies that compare protein levels between wild type plants and the VIM1 RING domain mutants using iTRAQ (isobaric tag for relative and absolute quantitation). In this experiment, nuclear protein from wild-type plants and *vim1 -/-* plants expressing the *VIM1* RING mutant constructs will be digested and labeled with different chemical tags. The samples will then be pooled and analyzed via nano liquid chromatography followed by tandem mass spectrometry. The two tags will generate distinct reporter ions when fragmented, allowing for relative quantitation of proteins between the samples. Significant abundance of a protein in the RING mutant plants relative to wild type may suggest that VIM1 participates in turnover of that protein, consistent with the fact that ubiquitination often targets proteins for proteasome-mediated degradation.

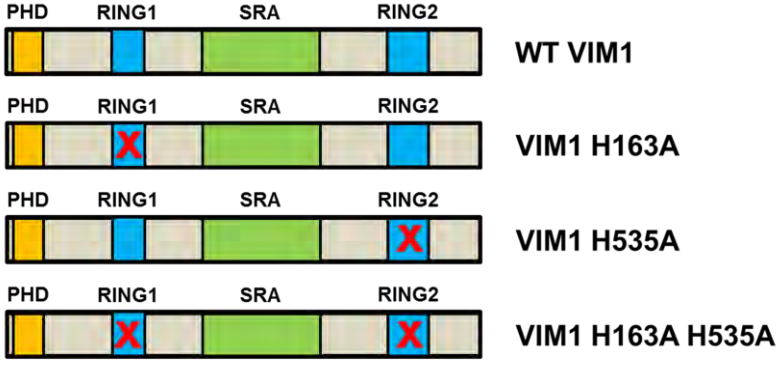


**Figure 2.** VIM1 point mutants expressed in a vim1 -/- background for comparative proteomics experiments. Each domain targeted for site-directed mutagenesis is indicated with a red X.

2. Confirm ubiquitination targets using an in vitro assay containing purified recombinant VIM1 (Months 1 – 36)

I have not yet completed the proteomics analysis described above, so there are no candidate substrates to test using an in vitro assay. However, I have developed an in vitro ubiquitination system that will be useful for these future experiments. In these assays, the required E1, E2, and E3 enzymes are combined in vitro under appropriate buffer conditions with other necessary components required for ubiquitination, including ubiquitin and ATP. Commercially available yeast UBE1, purified recombinant GST-UBC8, and purified recombinant GST-VIM1 act as the E1, E2, and E3 respectively. Using this system, I have been able to recapitulate results previously reported by another laboratory, demonstrating that VIM1 has E3 ubiquitin ligase activity in vitro[13]. In addition, I have used partial recombinant fragments of VIM1 to show that each of the two individual RING domains has activity (Fig. 3).

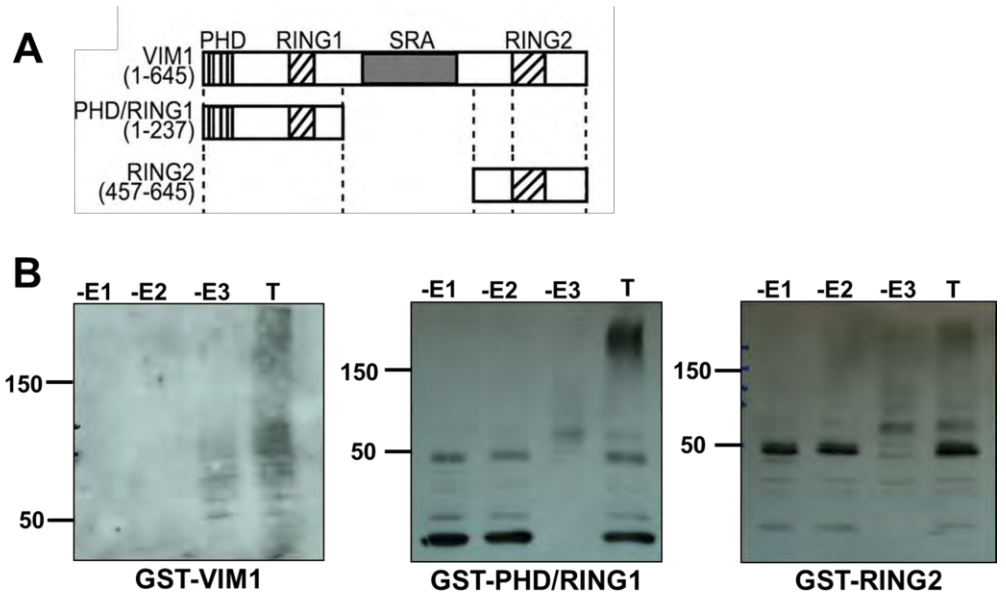


**Figure 3.** VIM1 RING domains have E3 ubiquitin ligase activity *in vitro*. A) Schematic representation of full-length and partial VIM1 proteins used in the ubiquitination assay. Length of each protein in amino acids is indicated. B) *in vitro* ubiquitination reactions lacking E1 enzyme (-E1), E2 enzyme (-E2), E3 enzyme (-E3), or containing all essential enzymes (T). The E3 protein used in each experiment is labeled at the bottom of the images. Ubiquitination reactions were run on a polyacrylamide gel, then immunoblotting was done against ubiquitin. The high molecular weight smear in the complete (T) reactions indicates the synthesis of polyubiquitin chains in vitro.

3. Assess the effects of RING domain mutations on DNA methylation and heterochromatin structure in vivo (Months 1 - 36)

My efforts to date in these experiments have been focused on generation of the RING domain point mutants described in Task 1, section 1 and Fig. 2. I will use the plant lines expressing these mutants to determine the relationship between VIM1 ubiquitin ligase activity and other epigenetic processes, such as maintenance of DNA methylation and heterochromatin structure. To examine DNA methylation, the Richards lab has previously used methylation-sensitive restriction digests combined with southern blotting against centromeric repeats (Fig. 4). Such an assay comparing DNA methylation between wild-type plants and those expressing VIM1 RING domain mutant proteins will provide insight on whether the RING domains are essential for VIM1's role in maintaining DNA methylation. Other chromatin marks, such as histone modifications, will also be compared between wild type and mutant plants via chromatin immunoprecipitation (ChIP). Additionally, differences in centromere structure between wild-type and VIM1 RING domain mutants will be examined via cytological techniques such as fluorescence in situ hybridization (FISH) against centromeric repeats. Previously, the Richards laboratory and colleagues reported that centromere structure

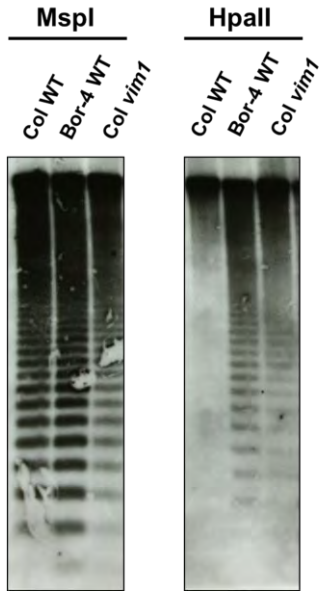


**Figure 4.** Arabidopsis *vim1* mutants are deficient in DNA methylation at centromeres. Genomic DNA was isolated from wild-type, *vim1* mutant, or Bor-4 plants and digested with isoschizomeric enzymes MspI (not sensitive) and HpaII, (methylation-sensitive), then blotted with a centromeric repeat probe. Ladder patterns in Bor-4 and *vim1* samples treated with HpaII suggest a loss of DNA methylation.

is significantly disrupted in *vim1 -/-* mutants, but it is unknown whether loss of RING domain activity plays a role in this phenotype[6].

**Task 2: Determine the *in vitro* specificities of the human UHRF1 RING and SRA domains**

1. Examine UHRF1 E3 ligase activity on human homologs of VIM1 substrates in vitro (Months 18 – 36)

These experiments have not been initiated.

2. Determine the methylcytosine-binding activity of the UHRF1 SRA domain using electrophoretic mobility shift assays (EMSA) (Months 1-24)

The purpose of this work is to closely examine the mechanism of methylcytosine binding in vitro, with special attention given to sequence context. Although it has been previously reported that the mouse homolog of UHRF1 specifically recognizes methylcytosines in CpG contexts[5], I intend to determine whether the human protein has the same specificity. The prospect of UHRF1 binding methylcytosine in non-CpG contexts is particularly intriguing, since the existence of non-CpG methylation has recently been reported in humans[14,15]. I have been working on these experiments together with Erika Hughes, a research technician in the Richards lab. Together, we have cloned and purified several UHRF1 partial proteins containing the SRA domain, and created several methylated double-stranded oligonucleotides for use as substrates (Fig. 5). We are currently optimizing the EMSA protocol using a commercial digoxigenin labeling and detection kit.

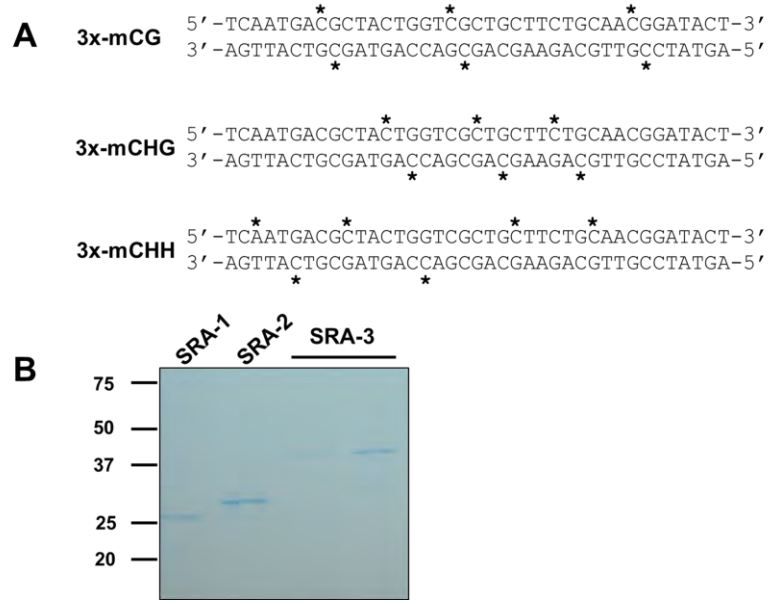


Figure 5. Reagents generated for EMSA experiments. A) Double-stranded oligonucleotide substrates bearing CG, CHG, or CHH substrates. The double-stranded oligos are identical aside from placement of the methyl groups. B) Purified recombinant His-tagged SRA domains. SRA-1 and SRA-2 are the same SRA domains used in previously published literature from other groups[5,16]. SRA-3 is an extended version.

**KEY RESEARCH ACCOMPLISHMENTS**

- Creation of plant lines expressing VIM1 RING point mutants under a native promoter in a *vim1 -/-* background. These will be useful for two of the experiments discussed in Task 1.
- Development of an in vitro ubiquitination assay for confirmation of candidate VIM1 substrates
- Confirmation of VIM1 E3 ubiquitin ligase activity in vitro, and demonstration that each of the two VIM1 RING domains independently has activity
- Purification of UHRF1 SRA domains and generation of oligonucleotide substrates for EMSA experiments

**REPORTABLE OUTCOMES**

- Poster presentation, “In vitro mechanism of UHRF1 methylcytosine binding,” at Era of Hope Conference, August 5 2011 (upcoming)

**CONCLUSION**

Though most of the experiments in this project are still ongoing, they have potential to provide information on fundamental epigenetic mechanisms. In particular, this work will uncover new substrates for the ubiquitin ligase activity of SRA-RING proteins, and further elucidate the specificities of their SRA domains. Understanding how these proteins function on the molecular level can eventually lead to the development of

new epigenetically based breast cancer therapies and diagnostic tools. Short-term efforts will focus on the proposed proteomics experiments for identification of new ubiquitinated substrates, confirmation of these substrates using in vitro assays, and optimization of the EMSA protocol.

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